

The study of cellular cytotoxicity of argireline® — an anti-aging peptide*

Marek Grosicki✉, Gniewomir Latacz, Annamaria Szopa, Anna Cukier and Katarzyna Kieć-Kononowicz

Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Kraków, Poland

Argireline® is well known, innovative anti-aging product used in the cosmetic market. This short chain peptide is used as active ingredient in dermal ointments and creams. Argireline® prevents formation of skin lines and wrinkles in a very similar way to the botulinum toxin (Botox), inhibiting neurotransmitter release at the neuromuscular junction. Argireline® does not require under skin muscle injections and it is believed to be relatively safe. However, despite the fact that some toxicity data has been provided by the product manufacturer, there is an evident lack of reliable information about cytotoxicity of argireline® in the literature. The aim of the presented study was to estimate the antiproliferation effect of argireline® solution in several concentrations. The influence of argireline® on cellular proliferation was examined against: human embryonic kidney HEK-293 cell line, human neuroblastoma IMR-32 cell line, and human primary skin fibroblasts. Tests were performed using formazan-based cell proliferation assay: EZ4U, which allows to measure the efficiency of mitochondrial oxidative activity in living cells. The argireline® inhibitory concentration, IC₅₀ values were calculated and the results were compared to the IC₅₀ value of the reference compound: doxorubicin. In conclusion, the considered method resulted in dose-dependent argireline® anti-proliferation effects. However, the significant cytotoxicity of argireline® solution was observed under 18 to 10 000 fold higher concentrations (depending on cells that were examined) in comparison to doxorubicin.

Key words: argireline, anti-aging peptides, cosmetology, cytotoxicity, antiproliferation

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INTRODUCTION

Over the last years cosmetic industry has been extensively developed towards biologically active products with drug-like effects. Cosmetic applied topically, such as creams, lotions and ointments can contain small biological active chemical compounds like: carbohydrates, lipids, antioxidants, peptides, and proteins (Schroeder, 2012). They are used in anti-solar, anti-aging, anti-wrinkle and firming products. Those active compounds help to improve appearance of aging skin by bettering its hydration, firmness and texture (Lupo *et al.*, 2007). Peptides are being incorporated in cosmetic formulas thanks to their wide biological effects. They play important role

in aging, inflammation, cell proliferation and migration, angiogenesis, protein synthesis and regulation, pigmentation, and in extracellular matrix synthesis (Zhang *et al.*, 2009). Cosmeceutical peptides are believed to be relatively safe, but the information about their cytotoxicity is still insufficient. Therefore the aim of presented study was to estimate the anti-proliferation effect of argireline® 0.05% solution, as an example of peptide used in cosmetic market.

Argireline® (acetyl hexapeptide-3, MW=889.1 g/mol) is a synthetic anti-aging peptide produced by Lipotec LTD. company (Neuronal exocytosis inhibiting peptides and cosmetic and pharmaceutical compositions containing said peptides, WO 00/64932, 2002, Lipotec LTD). In cosmetic products it is a biological active formulation ingredient, present most often in form of a powder or 0.05% Argireline® solution. Argireline® belongs to neurotransmitters inhibiting cosmeceutical peptide category. It has been derived from N-terminal end of SNAP-25 protein (aa 12–17) with following amino acid sequence: Ac-Glu-Glu-Met-Gln-Arg-NH₂ (Fig. 1).

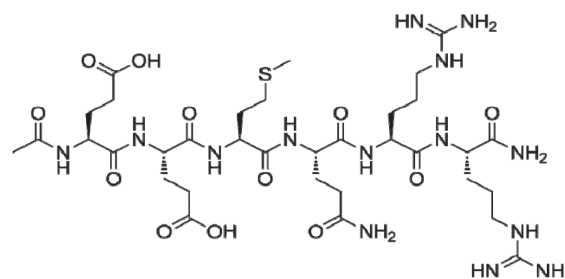


Figure 1. The chemical structure of argireline® (Blanes-Mira *et al.*, 2002).

Argireline® acts in similar way as botulinum neurotoxins (Botox®, Allergan, Irvine, CA) causing muscle paralysis by inhibiting activity in the presynaptic neuronal exocytosis machinery (Fields *et al.*, 2008). Active peptide inhibits skin muscle contraction, especially in the forehead, neck, nasal flare and around the eyes (Gorouhi *et al.*, 2009). Argireline® is blocking Ca²⁺ dependent neurotransmitter release (acetylcholine) at neuromuscular junction. This peptide is reacting with SNARE complex, consisting of three synaptic proteins SNAP-25, VAMP and syntaxin. Argireline® competes

✉ e-mail: grosickim@gmail.com

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Abbreviations: DX, doxorubicin; EZ4U, easy for you; HEK-293, human embryonic kidney; HSF, human skin fibroblast

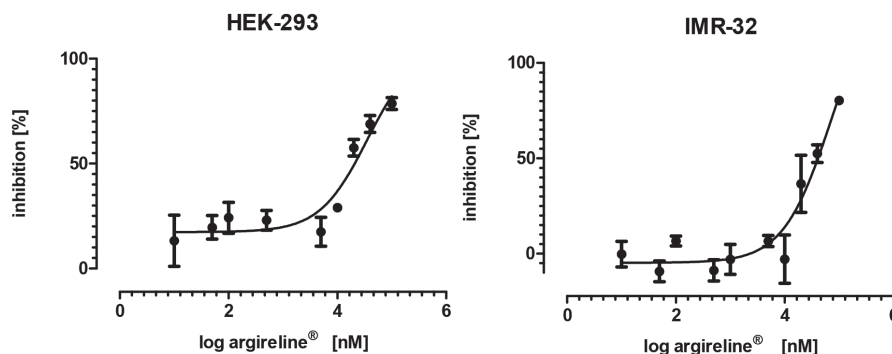


Figure 2. The antiproliferative effect of argireline® solution against HEK-293 (left) and IMR-32 (right) cell lines. Values represent the mean of $n = 4$ experiments.

with the SNAP-25 protein in the SNARE complex, essential for neuronal exocytosis, (Chen *et al.*, 2001). Due this complex is destabilized, preventing catecholamine and acetylcholine vesicle fusion with plasma membrane (Fields *et al.*, 2008). This peptide can significantly limit neurosecretion at micromolar concentration (Blanes-Mira *et al.*, 2002). The effectiveness of argireline® has been confirmed in several anti-wrinkle tests performed *in vitro* using chromaffin cells and *in vivo* on healthy volunteers. A clinical study published in the *International Journal of Cosmetic Science* reported that acetyl hexapeptide-3 at a 10% concentration in O/W emulsion reduces the depth of wrinkles up to 30% after 30 days of use (Blanes-Mira *et al.*, 2002). Although the anti-wrinkle effect of argireline® is now commonly known and well described in the literature, the cytotoxicity data of this compound was only found in commercial data sheets*. According to those data no signs of cytotoxicity were observed on human dermal fibroblasts and human epidermal keratinocytes, when argireline was administrated in concentrations between 10 µg/ml and 1 mg/ml. Also no genotoxicity was observed testing argireline® in concentrations ranging from 5 to 0.05 mg/plate in Ames test, performed on several *S. typhimurium* strains: TA97, TA98, TA100, TA 102 and TA 1537. The acute oral toxicity of argireline® has been also investigated. According to authors this small peptide exhibits its insignificant acute toxicity (2000 mg/kg) as compared with BTX-A (20 ng/kg) (Blanes-Mira *et al.*, 2002). Considering presented data we demonstrated independent study of antiproliferation effect of argireline® on human embryonic kidney HEK-293 cell line, human neuroblastoma IMR-32 cell line, and human primary skin fibroblasts. Experiments have been performed using formazan-based antiproliferation assay: EZ4U, which allows to measure the efficiency of mitochondrial oxidative activity in living cells. This test takes advantage of enzymatic bioreduction of tetrazolium salt into formazan product which can be quantified using spectrophotometric methods (Malich *et al.*, 1997; Krzysztos-Russjan *et al.*, 2009).

MATERIALS AND METHODS:

Reagents. Argireline® (MW=889,1g/mol) was kindly provided by Przedsiębiorstwo Produkcyjno-Handlowe Ryszard Kaczmarek i Synowie Sp. z o. o., Spółka Komandytowa, the official distributor of Lipotec S.A. prod-

ucts in Poland. Argireline was in form of 0.05% water solution. Concentration of pure active peptide in solution has been estimated around 0.56 mM, according to the information provided by manufacturer. Doxorubicin hydrochloride (2 mg/ml) was from Ebewe Pharma Poland. EZ4U Non-radioactive cell proliferation and cytotoxicity assay, was from Biomedica. For cell culture Dulbecco's Modified Eagle's Medium — DMEM (Gibco) was used, supplemented with fetal bovine serum (Gibco) and streptomycin/penicillin (Polfa Tarchomin).

Cell lines. Neuroblastoma IMR-32 cell line was provided by Department of Oncogenomics, Academisch Medisch Centrum, Amsterdam, Holland (Cheng *et al.*, 1995) (Tumilowicz *et al.*, 1970).

Human embryonic kidney HEK-293 cell line (ATCC CRL1573) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn).

Human skin fibroblasts (HSF) were kindly provided by Prof. Justyna Drukala (Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University). Human fibroblasts were isolated from skin graft, obtained from 38 years old male patient.

Cell culture. HEK-293 and IMR-32 cell lines, as well as human dermal fibroblasts were cultured in Dulbecco's Modified Eagle's complete growth Medium (DMEM); media contained 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 U/mL penicillin. Cells were cultured at 37°C in an atmosphere containing 5% CO₂.

In vitro proliferation assay using EZ4U. Cells were seeded in 96-well plates at a concentration of 2×10^4 cells/well (IMR-32), 1.5×10^4 cells/well (HEK-293) and 1×10^4 cells/well (HSF) in 200 µl culture medium and incubated for 24 h at 37°C and 5% CO₂ to reach 60% confluence. Next, argireline® was added into microplates at the final concentrations 0.01 µM–100 µM. The cells were incubated with argireline® for 48 h at 37°C and 5% CO₂. Then, 20 µl of EZ4U labeling mixture was added and the cells were incubated for 5 h under the same conditions. The absorbance of the samples was measured using a microplate reader (PerkinElmer) at 492 nm. The activity of the standard drug doxorubicin (DX) was estimated in the same way by using EZ4U at the concentrations 0.001–50 µM (against IMR-32) and at 0.005–100 µM (against HEK-293) and at 0.005–50 µM (against HSF). All experiments were conducted in four repetitions. GraphPad Prism 5.01 software was used to calculate the experimental IC₅₀ values.

*(www.lotioncrafter.com/pdf/argireline.pdf, 15.10.2013)

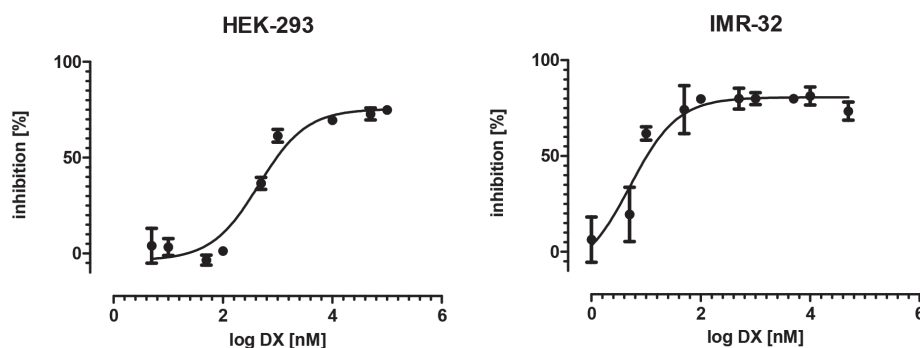


Figure 3. The antiproliferative effect of (DX) against HEK-293 (left) and IMR-32 (right) cell lines. Values represent the mean of n=4 experiments.

RESULTS AND DISCUSSION

Cytotoxicity tests, including formazan-based *in vitro* proliferation assays, are widely used in drug discovery research for testing safety of therapeutic agents before they are progressed to the clinic. The acceptable toxicity as well as the other parameters such as: absorption, distribution, metabolism, elimination, describe the ability of the compound to be an ideal drug candidate (Kerns *et al.*, 2008). Additionally, *in-vitro* toxicity testing is a reliable alternative to the animals toxicity testing by reduction, replacement and refinement (3Rs) approach (Smith 2001). Argireline® is the peptide with very high biological activity and therefore should be considered not only as an anti-wrinkle ingredient used in cosmetology but also as a potential toxic agent in case of long-term skin exposure or overdose. That justify the necessity of its safety testing just as any other drug. The incubation of argireline® solution in different concentrations with human embryonic kidney HEK-293 and neuroblastoma IMR-32 cell lines for 48 h had dose dependent antiproliferative effect (Fig. 2). In lower concentration no difference between argireline® and the control without active peptide was observed on proliferative activity of the examined cells. However, the significant antiproliferative activity was observed above 10 μ M argireline® concentration. The calculated IC_{50} values were 34.862 μ M against HEK-293 and 68.458 μ M against IMR-32 respectively.

The antiproliferative effect of the commonly used drug in cancer chemotherapy doxorubicin (DX) on HEK-293 and IMR-32 was also examined. This compound is used in anticancer treatment and possesses well

examined antiproliferative effects. Therefore, it is commonly used as a standard compound in comparison of cytotoxic properties of unknown substances. The obtained data showed very strong antiproliferative effect of DX against neuroblastoma cell line IMR-32 (IC_{50} = 0.0051 μ M) and human embryonic kidney HEK-293 cell line (IC_{50} = 0.455 μ M) (Fig. 3). Comparing the results of antiproliferative effect of argireline® and doxorubicin (standard compound), it has to be pointed out that the IC_{50} value of argireline® is almost 75 fold higher than IC_{50} of DX against HEK-293 cell line and more than 10000 fold higher against IMR-32 cell line.

Argireline® is a synthetic anti-aging peptide, applied topically, therefore we also examined its anti-proliferative effect on human skin cells. Argireline® solution incubated in different concentrations with HSF had a dose-dependent antiproliferative effect on those cells (Fig. 4).

Cellular proliferation was not effected in argireline® low concentration. The evident antiproliferative effect (67% inhibition) has been observed at 100 μ M. The antiproliferative effect of DX against HSF was also examined. The results are shown in Fig. 5.

Contrary to HEK-293 and IMR-32, doxorubicin was less cytotoxic against HSF, effecting these cells above 1 μ M (IC_{50} = 5.628 μ M). In the comparison with other examined cells, the antiproliferative effect of DX against HSF was more than 700 fold lower for IMR-32 (IC_{50} = 0.0051 μ M) and 8 fold lower for HEK-293 (IC_{50} = 0.455 μ M). Comparing the results of the antiproliferative effect of argireline® and doxorubicin against HSF, the significant effect of argireline® was observed at 100 μ M, whereas for doxorubicin at 18 fold lower concentration

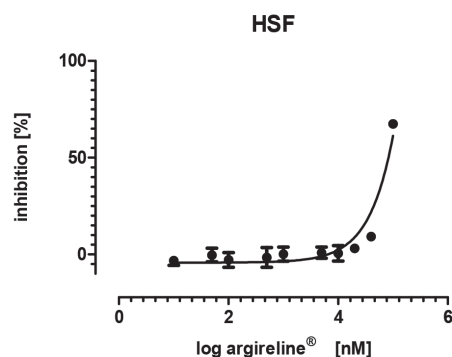


Figure 4. The antiproliferative effect of argireline® solution against human skin fibroblasts (HSF). Values represent the mean of n=4 experiments.

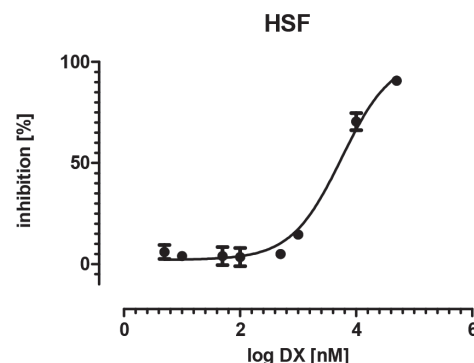


Figure 5. The antiproliferative effect of doxorubicin (DX) against human skin fibroblasts (HSF). Values represent the mean of n=4 experiments.

5.628 μM . However, argireline[®] producer states that no signs of peptide cytotoxicity were observed on human dermal fibroblasts at concentrations between 10 $\mu\text{g}/\text{ml}$ and 1 mg/ml *. This was not confirmed in our experiments, where argireline exhibited 67% antiproliferative effect after 48 h of incubation at 100 μM (90 $\mu\text{g}/\text{ml}$). Unfortunately, the detailed comparison study is impossible due to unpublished data about the experiments which were undertaken by manufacturer. Therefore, further antiproliferation study should be also performed to verify the manufacturer results for human epidermal keratinocytes. In conclusion, this comparative study reveals that argireline[®] seems to be a safe compound in compare to the reference compound doxorubicin. Notwithstanding this fact, taking to the account the cytotoxic activity against human skin fibroblasts the safety profile of this product is of concern and the use of argireline[®] in very high doses or for a very long period of time must be considered as potentially dangerous for patients.

Acknowledgements

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REFERENCES

- Blanes-Mira C, Clemente J (2002) A synthetic hexapeptide (Argireline) with antiwrinkle activity. *Int J Cosmet Sci* **24**: 303–310.
- Cheng NC, Van Roy N, Chan A, Beitsma M, Westerveld A, Speleman F, Versteeg R (1995) Deletion mapping in neuroblastoma cell lines suggests two distinct tumor suppressor genes in the 1p35-36 region, only one of which is associated with N-myc amplification. *Oncogene* **10**: 291–297.
- Chen YA, Scheller RH (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol* **2**: 98–106.
- Gorouhi F, Maibach HI (2009) Role of topical peptides in preventing or treating aged skin. *Int J Cosmet Sci* **31**: 327–345.
- Kerns EH, Di L. (2008) *Drug-like properties: Concepts, structure design and methods*. Academic Press, Burlington, MA.
- Krzysztoń-Russjan J, Książek I, Anuszevska E (2009) Porównanie użyteczności testów MTT i EZ4U stosowanych do oceny cytotoksyczności ksenobiotykw. *Farm Pol* **65**: 395–402 (in Polish).
- Lipotec S.A. Neuronal exocytosis inhibiting peptides and cosmetic and pharmaceutical compositions containing said peptides. *European patent*. WO 00/64932, 2002.
- Lupo MP, Cole AL (2007) Cosmeceutical peptides. *Dermatol Ther* **20**: 343–349.
- Malich G, Markovic B, Winder C (1997) The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. *Toxicology* **124**: 179–192.
- Smith R (2001) Animal research: the need for a middle ground: Let's promote the three Rs of animal research: replacement, reduction, and refinement. *BMJ: Brit Med J* **322**: 248.
- Tumilowicz JJ, Nichols WW, Cholon JJ, Greene AE (1970) Definition of a continuous human cell line derived from neuroblastoma. *Cancer res*, **30**: 2110–2118.
- Zhang L, Falla TJ (2009). Cosmeceuticals and peptides. *Clin Dermatol* **27**: 485–494.